

# Influence of Dietary Soy Phytoestrogens on Cytokine Production and Immunoglobulin in Ovariectomized Rats

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**Abstract**—This study was designed to examine the relationship between soy phytoestrogens supplementation in ovariectomized rats and pro-inflammatory cytokines Interleukin 1 alpha (IL-1 $\alpha$ ), Interleukin 2 (IL-2), Interleukin 4 (IL-4) and immunoglobulin. Twenty four female rats were ovariectomized then divided equally into 3 groups; control group, was fed on casein based diet, low soy phytoestrogens group was fed a diet contained 7% soy and high soy phytoestrogens group received a diet of 26% soy for 28 days. At the end of experiment, rats were weighed then blood samples were collected on EDTA for total leukocyte count (TLC). Serum samples were taken for interleukins assay, lysozyme activity and immunoglobulin. Animals were sacrificed and thymus and spleen were weighed. The body weight gain was significantly declined in ovariectomized rats fed high soy diet at the first 3 weeks of the experiment. Rats fed high soy diet showed significant reduction in thymic weight compared to control ones. IL-1 $\alpha$  and IL-2 were significantly suppressed in rats fed high soy diet compared to control rats. However, IL-4 was significantly stimulated in rats fed high soy diet compared to control rats. Both IgM and IgG levels were significantly increased in rats fed high soy diet compared to control rats. There was a significant decrease in lysozyme activity in rats fed high soy diet compared to rats fed low soy diet. TLC was significantly increased in rats fed high and low soy diets. In conclusion, soy supplementation altered selected immunological parameters of ovariectomized female rats.

**Keywords**—Soy, ovariectomized rats, interleukins, immunoglobulin.

## I. INTRODUCTION

IN recent years, researchers have tried to unravel the highly complex relationship between nutrition, immunology and cancer. A growing body of evidence suggests that nutrition and immune function are closely related. The cells of the immune system evolve from pluripotent hematopoietic stem cells found in the bone marrow. The immune cells are classified as either granulocytes or agranulocytes. The granulocytes include the basophils, mast cells, eosinophils, and neutrophils and the agranulocytes include the monocytes, natural killer (NK) cells, and lymphocytes. The lymphocytes

are further divided into the B lymphocytes and T lymphocytes [1]. Natural killer (NK) cells which make up about 15% of circulating lymphocytes can be also distinguished by their unique cell surface markers. Resting NK cells also express a portion of certain cytokine receptors such as the interleukin-2 receptor [2]. In turn, cytokines represent a group of molecules, which are produced by T cells, B cells and/or macrophages to serve as mediators between the different types of immune cells. For example, binding with IL-2 leads to stimulation and subsequent activation of NK cells and T cells [3]. Additionally, immunoglobulin M (IgM) is the first antibody to respond to foreign antigen. While, immunoglobulin G (IgG) accounts for 70-75% of the immunoglobulin in human serum. It is the major antibody of the secondary immune response [1].

Soybeans are rich sources of the phytoestrogens genistein and daidzein (belong to isoflavones), which continue to be investigated for their potential in reducing the risk for cardiovascular diseases, osteoporosis and certain types of cancer [4]. Soy phytoestrogens may affect immune functions as weak estrogen agonists or antagonists. Both genistein and daidzein possess weak estrogenic activity due to their structural similarity to 17 $\beta$ -estradiol [5]. Since the immune system is, in part, regulated by steroid hormones [6], soy phytoestrogens may influence immunocompetence via estrogen-receptor mediated mechanisms. In addition, soy phytoestrogens possess a variety of immune characteristics including antioxidant, anti-proliferative and anti-inflammatory properties.

Despite the hypothesized beneficial effects of phytoestrogen, there are concerns about the potential side effects of this compound on immune system in animals. Therefore, this study was designed to examine the relationship between soy phytoestrogens supplementation in ovariectomized rats and specific aspects of immunity as pro-inflammatory cytokines (IL-1 $\alpha$ ), preferential production of Th1 type of cytokines (IL-2), Th2 cytokine regulatory pathway (IL-4) and immunoglobulin.

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## II. MATERIALS AND METHODS

### A. Animals and Ovariectomy

Twenty-four adult female Albino rats weighing approximately 100-110 g were purchased from Lab. Animal House, Faculty of Veterinary Medicine, Suez Canal University. They were housed under standard laboratory conditions and kept at room temperature ( $28 \pm 2$  °C) with free access to casein based diet (soy free) and water. Experiment was carried out under Animal Care and Use Protocols Procedures approved by Faculty of Veterinary Medicine, Suez Canal University Committee, Egypt.

Female rats were ovariectomized (OVX), 2 week after arrival under effect of diethyl ether inhalation anesthesia [7]. Rats were given amoxicillin 10 mg/ kg orally for 5 successive days after ovariectomy as well as casein based diet and water *ad libitum* and maintained on 12 h day and 12 h night cycle.

### B. Experimental Groups

Twenty days after ovariectomy, the OVX female rats were divided randomly into three groups; control group (n= 8) was fed on casein based diet (soy free diet), low soy phytoestrogens group (n= 8) was fed a diet contained 7% soy and high soy phytoestrogens group (n= 8) received a diet of 26% soy for 28 days. All diets were formulated according to Tag *et al.* [8] to fulfill all the nutritional requirements of adult rats according to NRC [9]. Food consumption for all rats in each group/week was determined. Body weight gain (g/week) was obtained by determination of difference between two successive body weights, for each rat at one-week interval.

### C. Blood and Tissue Samples

At the end of experiment (28 days of treatment), the rats were weighed then blood samples were collected from retro-orbital venous plexus on EDTA tubes for total leukocyte count. Other blood samples were taken in plain tubes and were kept for 15 min. at room temperature then centrifuged at 3000 rpm for 20 min. The serum was stored at -20 °C till interleukins assay, immunoglobulin and lysozyme assay. Animals were then sacrificed. Thymus and spleen were dissected and weighed. Their relative weights were calculated in relation to body weight as follow,

Relative thymic weight = [thymus weight (g) / body weight (g)] X 100

Relative splenic weight = [spleen weight (g) / body weight (g)] X 100

### D. Interleukins Assay

Serum IL-1 $\alpha$  was analyzed by ELISA using solid phase sandwich ELISA obtained from Biological Laboratories Co. Ltd. (Catalog No. 27193, USA). IL-2 was measured using specific rat ELISA kits (IB49713, USA). IL-4 was measured using specific rat ELISA kit (IB49709, USA). All parameters were determined according to kit manufacturer instruction.

### E. Immunoglobulin Assay

IgM and IgG assays were performed by Abcam's rat ELISA Kit (KEMET Medical, Egypt). Abcam's rat ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of IgM and IgG. In this assay the IgM present in samples reacts with the anti-IgM antibodies, which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-IgM antibodies conjugated with horseradish peroxidase (HRP) are added. These enzyme-labeled antibodies form complexes with the previously bound IgM. Following another washing step, the enzyme bound to the immunosorbent is assayed. The absorbance, at 450 nm, is a measure of the concentration of IgM in the test sample.

### F. Lysozyme Enzyme Assay

Lysozyme activity was measured by agarose gel cell lysis assay according to the method described by Schultz [10]. Briefly, lysoplates were prepared by dissolving 0.01% agarose in 0.0067 M PBS (pH 6.3) at 100 °C, when agarose temperature reached 60-70 °C, 500 mg of uniform suspension of *Micrococcus lysodeikticus* in 5 ml saline was added to one liter of agarose and mixed well, then the plates were poured into wells. To which, exactly 25  $\mu$ l of each of the serum samples and standard lysozyme solutions were put in each poured well. They were incubated at 28-30 °C for 12-18 h and the clear zone ring diameters were measured. The diameters of clear zones were plotted against the standards for obtaining lysozyme concentration in the samples.

### G. Total Leukocyte Counts (TLC)

Blood samples were collected from retro-orbital venous plexus of ovariectomized female rats at the end of experimental period on EDTA containing tubes and placed immediately on ice for TLC. Leukocytes were counted by Neubauer's improved haemocytometer using Turkey's solution. The number of leukocytes was calculated according to Feldman *et al.* [11].

### H. Statistical Analysis

All values were expressed as mean  $\pm$  standard error of the mean. One-way analysis of variance was used for analyzing the differences among groups. Inter-group comparisons were made by Tukey's test. *P* value of < 0.05 was considered to indicate significance. All the analyses were done using Graph Pad Prism (Version 5.01, Graph Pad software, San Diego, USA).

## III. RESULTS

### A. Body Weight Gain and Food Intake

The body weight gain was significantly declined in ovariectomized rats fed high soy diet at the first three weeks of the experiment. However, no significant difference was detected in the body weight gain at the end of the experiment

(Fig. 1). The food intake was similar in control and treated female rats without any significant differences.

### B. Spleen and Thymus Weights

Rats fed high soy diet showed significant reduction in thymic weight compared to that of control ones. While, there was no difference in splenic weight between control and treated rats (Fig. 2).

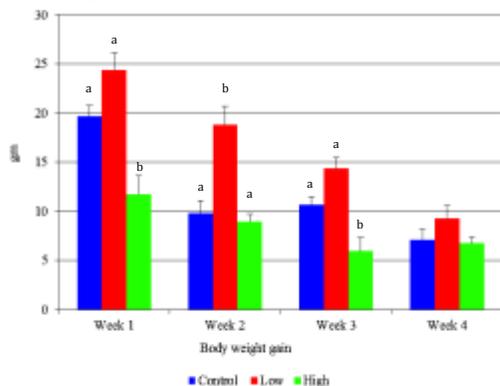


Fig. 1: Body weight gain at different weeks in ovariectomized rats fed low and high soy diets

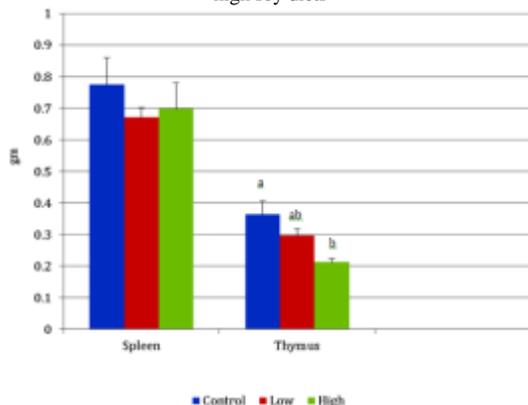


Fig. 2: Spleen and thymus weights in ovariectomized rats fed low and high soy diets

### C. Interleukin Assay

IL-1 $\alpha$  and IL-2 were significantly suppressed in rats fed high soy diet compared to that in control rats. However, IL-4 was significantly stimulated in rats fed high soy diet compared to that in control rats (Table 1).

### D. Immunoglobulin

Both IgM and IgG were significantly increased in rats fed high soy diet compared to that in control rats (Table 1).

### E. Lysozyme Activity Assay and TLC

There was a significant decrease in lysozyme activity in rats fed high soy diet compared to rats fed low soy diet. TLC was significantly increased in rats fed high and low soy diet compared to that in control rats (Table 1).

TABLE I: EFFECTS OF SOY SUPPLEMENTATION ON INTERLEUKINS, IMMUNOGLOBULIN, LYSOZYME ACTIVITY AND TOTAL LEUCOCYTE IN CONTROL, LOW AND HIGH SOY DIET FED RATS.

	Control	Low (7% soy)	High (26% soy)	P value
IL-1 $\alpha$ (pg/ml)	8.69 $\pm$ 0.22*	5.69 $\pm$ 0.27*	3.35 $\pm$ 0.12*	0.0001
IL-2 (pg/ml)	0.88 $\pm$ 0.03*	0.77 $\pm$ 0.03*	0.69 $\pm$ 0.02*	0.0007
IL-4 (pg/ml)	33.03 $\pm$ 2.47*	46.23 $\pm$ 1.48*	93.98 $\pm$ 8.36*	0.0001
IgG (mg/ml)	8.02 $\pm$ 0.37*	10.46 $\pm$ 0.32*	12.03 $\pm$ 0.45*	0.0001
IgM (mg/ml)	0.24 $\pm$ 0.01*	0.28 $\pm$ 0.01*	0.33 $\pm$ 0.019*	0.002
Lysozyme activity (mg/ml)	288.3 $\pm$ 1.06**	321.4 $\pm$ 9.44*	277.3 $\pm$ 14.8*	0.03
TLC $\times$ 10 <sup>3</sup> / $\mu$ l	10223 $\pm$ 541.4*	15038 $\pm$ 866.4*	18912 $\pm$ 578.3*	0.0001

Different superscripts within the same row indicated significant differences at  $P < 0.05$

## IV. DISCUSSION

Data from animal studies have indicated that phytoestrogens may either enhance or suppress immunocompetence depending on their concentration, the target tissue and a number of other factors. The mechanisms underlying these effects are not fully understood, and further investigations are needed to explore the detailed molecular mechanism regulated by phytoestrogens. Genistein significantly reduced inflammatory mediators, such as IL-1 $\beta$ , IL-6 and IL-8 production, in TNF- $\alpha$ -activated MH7A cells *in vitro* [12]. The anti-inflammatory properties of genistein are mediated by the interruption of the ROS/Akt/NF- $\kappa$ B signaling pathway in MH7A cells. These signaling led to a marked decrease of TNF- $\alpha$ -induced pro-inflammatory cytokine production [12].

In the present study, the body weight gain was significantly declined in ovariectomized rats fed high soy phytoestrogens diet at the first three weeks of the experiment. However, no significant difference was detected in the body weight gain at the end of the experiment. Additionally, there were no significant changes in food intake in control and treated rats. The decrease in body weight gain in spite of absence of change in food consumption may be due to loss of large amount of ingested food energy during the increased locomotor activity [13], as observed in the rats of the current study. This decrease suggested that estrogenic hormone action of phytoestrogens was beneficial to body fat regulation and influenced the hypothalamic neuropeptide levels [14, 15].

In the present study, rats fed high soy diet showed significant reduction in thymic weight compared to that of control ones. Genistein (5 mg/d for 21 d) intravenously injected into ovariectomized female mice resulted in an 80% reduction of thymic weight, while genistein injection at 2 mg/d for only 7 d led to a 70% decrease in thymic weight [16]. Genistein reduced the numbers of peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells and this reduction might come from thymic atrophy [17].

In the present study, there was a stimulation of IL-4 and suppression of IL-1 $\alpha$  and IL-2 levels in ovariectomized rats fed high soy diet. This may indicate that phytoestrogen may inhibit and/or stimulate specific immune cells. Possible link

between high levels of soy consumption and immune impairments was contradictory. There were previous reports indicated that T cell function and immunoglobulin were decreased in infants fed soy formula compared with cow milk formula fed controls [18, 19]. Further evidence implies that soy phytoestrogens may suppress immune function when rats fed diets containing high doses of soy phytoestrogens as immunosuppressive agent to delay rejection of rat cardiac allografts [2]. In contrast, two reports did not confirm the extensive immune abnormalities described previously and reported that a number of parameters related to immune cell populations were within the normal range in soy-fed infants [20, 21]. Phytoestrogen and some of their metabolites may affect allergic responses via the enhancement of IL-4 production in T cells [22]. The functions of T cells and NK cells were altered after oral exposure to genistein at physiologically relevant concentrations in experimental animals. Specifically, exposure to genistein in adult female mice for 28 days by gavage increased the activities of cytotoxic T cells and NK cells [23].

Both IgM and IgG were significantly increased in rats fed high soy diet compared to that in control rats. Although genistein increased IgM at the dose of 6 mg/kg in adult female B6C3F1 mice, no effect was observed in the high dose group. Furthermore, exposure to genistein had no effect on IgG response [23]. Oral administration of daidzein to Swiss mice increased the thymic mass and number of lymphocytes in blood and resulted in elevated antigen-specific IgM [24]. In the current study, it was clear that soy phytoestrogens stimulated the B1 cell response which is immunocompetent and suppressed T cell response. This confirmed the estrogenic effect of soy as estrogen is well known to stimulate IgM from B1 cells by direct way through ER- $\beta$  signaling pathway [25].

In the present work, the increase in IL-4, IgG and IgM as well as the decrease in pro-inflammatory cytokines IL-1 $\alpha$  and IL-2 implies the favorable effect of soy phytoestrogens on Immunity as they improved general humeral immune response and prevented autoimmunity as IL-4 stimulated signaling array in B cells [26]. Moreover, inhibition of IL-2 production led to the inhibition of Th1 and enhancement of Th2 cytokine synthesis in CD4+ T cells [27].

There was a significant decrease in lysozyme activity in rats fed high soy diet compared to rats fed low soy diet. Lysozyme is a ubiquitous enzyme present in biological secretions and leukocyte. Lysozyme hydrolyzes  $\beta$ -(1-4)-glucosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of a variety of microorganisms. It might reflect the effect of soy on phagocytic ability of the macrophage in ovariectomized rats. In the preset study, TLC was significantly increased in rats fed high and low soy diet compared to that in control rats. This might highlight the role of estrogen in down regulating the expression of adhesion and chemokine molecules in response to inflammation [28]. Researchers have also reported that estrogen treatment altered the recruitment and adhesion of leukocytes to the

endothelium, which was induced by inflammation [29]. The result of TLC and lysozyme enzyme activity supported the anti-inflammatory effect of soy phytoestrogens and their promising rule in prevention of autoimmune diseases.

## V. CONCLUSION

Data from this study revealed that 28 days of soy phytoestrogens supplementation altered selected immunological parameters in ovariectomized rats. This is important information proved the ability of the soy phytoestrogens to modulate immunoglobulin and cytokine production in ovariectomized female rats by favoring the humeral immunity and lowering auto-immunity. However, further studies documenting the long-term effect of soy supplementation on immunity are warranted.

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